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INTRODUCTION

During development, peripheral neurons become dependent on target-derived neurotrophins for survival and maintenance of differentiated functions. Failed or inappropriate target interactions *in vivo*, or withdrawal of neurotrophins *in vitro*, lead to a characteristic sequence of molecular cell death events termed "apoptosis". The purpose of the proposed research is to examine the roles of the *Nf1* gene product, neurofibromin, in modulating the apoptotic response to neurotrophin withdrawal, as well as the survival response to depolarization. We have shown that many sensory neurons isolated from *Nf1*^{-/-} mouse embryos survive in the absence of neurotrophins (Vogel et al., 1995). The scope of the experiments proposed for Year 1 included 1) comparison of the response of embryonic mouse sensory neurons to nerve growth factor withdrawal and subsequent rescue with depolarizing levels of KCl, 2) analysis of the roles of downstream signaling molecules (PI3 kinase, MAP kinase) in regulating the survival response to depolarization, and 3) analysis of the response of neurofibromin-deficient (*Nf1*^{-/-}) sensory neurons to neurotrophin withdrawal.

BODY

Task 1. Characterize responses of *Nf1*^{+/+} and *Nf1*^{+/-} DRG and retinal neurons to activity-mediated survival signaling *in vitro*. (Months 0-6).

DRG Neurons. During the first year of funding, we characterized survival responses of embryonic mouse dorsal root ganglion (DRG) sensory neurons to different concentrations of depolarizing KCl, following nerve growth factor (NGF) withdrawal at 24, 48, or 72 hours *in vitro*. Levels of intracellular calcium and potassium are known to influence apoptosis, particularly in postmitotic neurons (reviewed by Yu et al., 2001). In pheochromocytoma cells, a model for sympathetic neurons, membrane depolarization induced by high extracellular potassium leads to calcium influx through L-type voltage-sensitive calcium channels; this calcium influx stimulates growth factor receptor signal transduction (Rosen and Greenberg, 1996). Because we know that loss of neurofibromin leads to neurotrophin-independent survival of sensory neurons, due to increased levels of activated Ras (Klesse and Parada, 1998; Vogel et al., 2000), and because calcium signaling can regulate Ras activity (reviewed by Cullen and Lockyer, 2002), we proposed that *Nf1* gene dosage would influence the survival response to depolarization. Our hypothesis was that neurons with lower amounts of neurofibromin (*Nf1*^{+/-}) would be more sensitive to the survival-promoting effects of high potassium than would wild-type (*Nf1*^{+/+}) neurons, due to higher levels of activated Ras. For Task 1, our experiments with E13, E15, and E17 *Nf1*^{+/+} and *Nf1*^{+/-} DRG neurons support this hypothesis. However, a demonstrable difference in survival between *Nf1*^{+/+} and *Nf1*^{+/-} DRG neurons appears to depend on at least two factors: 1) the embryonic stage of the isolated neurons, and 2) the time *in vitro* at which NGF is withdrawn from the cultures and replaced with KCl. **Figures 1A and 1B** show survival of E13 DRG neurons following NGF withdrawal at 24 and 72 hours *in vitro*, respectively. No significant differences in E13 DRG neuron rescue by either 40 or 50 mM KCl were apparent for *Nf1*^{+/+} and *Nf1*^{+/-} cultures following NGF withdrawal at 24 hours (**Figure 1A**). In contrast, slight (but significant and reproducible) differences between survival of *Nf1*^{+/+} and *Nf1*^{+/-} E13 neurons in high K⁺ were observed following NGF withdrawal at 72 hours *in vitro* (**Figure 1B**). For E15 DRG, we observed significant differences between survival of *Nf1*^{+/+} and *Nf1*^{+/-} neurons following NGF withdrawal at 48 and 72 hours, at concentrations of 20, 30, and

40 mM KCl (**Figures 2A and 2B**). Similarly, reproducible differences between *Nf1*^{+/+} and *Nf1*^{+/-} E17 DRG neurons were observed in 40 or 50 mM KCl following NGF withdrawal at 24 or 72 hours *in vitro* (**Figures 3A and 3B**). It should be emphasized that all neuronal counts are performed by an investigator (K.Vogel) who is "blind" to the genotypes until the experiment is completed.

An unexpected outcome of the NGF withdrawal experiments for all three embryonic stages (E13, E15, E17) was that in the cultures that received no KCl, apoptosis occurred slightly more rapidly for *Nf1*^{+/+} than for *Nf1*^{+/-} neurons (**Figures 1, 2, 3**). For sympathetic superior cervical ganglion (SCG) neurons, we observed differences in neurotrophin-independent survival between those isolated from *Trp53*^{+/+} and *Trp53*^{+/-} mouse embryos at several different stages (Vogel and Parada, 1998), but we have not previously demonstrated such differences for *Nf1*^{+/+} and *Nf1*^{+/-} DRG neurons. Although our survival assays are sensitive, and involve counts of the same populations of neurons on successive days *in vitro*, the NGF withdrawal paradigm may be required to reveal differences between wild-type and *Nf1* haploinsufficient neurons. **In conclusion, we have demonstrated significant, reproducible differences in both apoptosis rate and survival response to depolarizing levels of potassium, between DRG neurons isolated from *Nf1*^{+/+} and *Nf1*^{+/-} mouse embryos at E13, E15, and E17.** We anticipate that these results, in combination with those we obtain from the experiments in Task 2 (in progress), will form the basis for a manuscript to be submitted to the Journal of Neuroscience.

Although there is a great deal of literature on the survival-promoting effects of depolarizing potassium levels for different types of neurons, our experiments differ from those published to date in several ways. First, the majority of such studies are performed with neurons (usually cerebellar or SCG) isolated at neonatal or postnatal stages; for our studies, we have used DRG sensory neurons isolated from mouse embryos. Our initial experiments on the role of neurofibromin in survival signaling focused on embryonic sensory neurons (Vogel et al., 1995), and subsequent analyses of signaling pathways (Klesse and Parada, 1998) utilized DRG sensory neurons isolated prior to E14, when *Nf1*^{-/-} mouse embryos die (Brannan et al., 1994). Second, in contrast to most published studies, our experiments compare the responses of neurons isolated from three different developmental stages (E13, E15, E17). Such comparisons are critical to understanding the interactions between signaling pathways, since both the spectrum of voltage-sensitive calcium channel expression and the sensitivity to neurotrophin dosage and withdrawal change during development (Hilaire et al., 1996; Carey and Matsumoto, 1999; Schmid and Guenther, 1999; Davies 2003). Third, the existence of a targeted mutation in the *Nf1* gene has allowed us to compare the responses of neurons isolated from mouse embryos of two different genotypes: *Nf1*^{+/+} and *Nf1*^{+/-}, and to demonstrate significant differences in the response to NGF withdrawal. On the other hand, this third difference also allows us to interpret our data in the context of reports that indicate an effect of *Nf1* haploinsufficiency on the behavior of other cell types, including astrocytes (Gutmann et al., 2001), melanocytes (Ingram et al., 2000; Kemkemer et al., 2002), mast cells (Ingram et al., 2000), fibroblasts (Rosenbaum et al., 1995; Atit et al., 1999), and Schwann cells (Kim et al., 1997).

Retinal Neurons. We experienced serious difficulties with these experiments, primarily because for both E13 and E15 retinal cultures, neurons continued to differentiate throughout the first 4 to 6 days *in vitro* (**Table 1**). This became especially apparent and problematic once we had

optimized the dissection, dissociation, and neuron culture parameters for embryonic retinae. The retinal neurons extend long axons on the laminin substratum and express several differentiated markers, even in the absence of brain-derived neurotrophic factor (BDNF; **Table 2**). Moreover, after a brief recovery phase, BDNF withdrawal and subsequent addition of KCl appear to elicit a new "wave" of neuronal differentiation in the retinal cultures. I propose two potential solutions to this problem: 1) restrict the experiments to retinal neurons isolated from neonatal or postnatal mice, and 2) perform the experiments with either trigeminal sensory or SCG sympathetic neurons isolated from E13, E15, and E17 *Nf1*^{+/+} and *Nf1*^{+/-} mouse embryos. Regarding alternative #2, the use of SCG sympathetic neurons offers advantages because the responses to NGF withdrawal have been characterized in great detail, at least for neonatal rat SCG neurons; however, the role of neurofibromin in regulating these responses is unknown. On the other hand, our own results with exon-specific *Nf1* knockout mice (*exon23a*^{-/-}) indicate that trigeminal sensory neurons may be especially sensitive to neurofibromin dosage, in the context of modulating neurotrophin responsiveness. In any case, I will await the recommendations of the reviewers of this annual report before I alter the experimental design for any of the Tasks.

Task 2. Identify signal transduction pathways activated by KCl-mediated depolarization in DRG and retinal neurons. (Months 6-12)

These experiments are in progress for E15 and E17 DRG neurons with inhibitors of PI3 kinase (LY294002 and wortmannin). Since we have optimized the NGF withdrawal and KCl rescue conditions, we expect to complete these experiments within the next 4-6 months without complications, and to combine the results with those obtained for DRG neurons in Task 1 for a manuscript.

Task 3. Characterize synergy of neurotrophin- and activity-mediated survival signaling in *Nf1*^{+/+} and *Nf1*^{+/-} DRG and retinal neurons. (Months 12-18) N/A

Task 4. Characterize the role of neurofibromin in mediating neuronal apoptosis following neurotrophin withdrawal. (Months 0-6)

DRG Neurons. During the first year of funding, we characterized the response of E12.5 *Nf1*^{-/-} DRG neurons to NGF withdrawal and KCl rescue. Based on our previous results (Vogel et al., 1995), we predicted that *Nf1*^{-/-} DRG neurons would not undergo apoptosis following NGF withdrawal, whereas neurons isolated from *Nf1*^{+/+} and ^{+/-} littermates should die 24-48 hours after NGF is removed. To our surprise, we found that approximately 50% of *Nf1* mutant E12.5 DRG neurons undergo apoptosis within 24-48 hours of NGF withdrawal (**Figure 4**); however, this percentage does not increase significantly over the subsequent 3-4 days in culture (data not shown). Young (E12.5) DRG neurons isolated from *Nf1*^{+/-} and ^{+/+} littermates appear to be very sensitive to the removal of survival factors, and the majority have undergone apoptosis within 24 hours of NGF removal (**Figures 4 and 6**). In contrast to the loss of many *Nf1*^{-/-} neurons following NGF removal, we did not observe apoptosis in sister cultures of neurofibromin-deficient neurons that had never been exposed to NGF *in vitro* (**Figure 4**). This is consistent with our previous data with both DRG and SCG neurons (Vogel et al., 1995), and raises the intriguing possibility that neurotrophin exposure elicits dependence (Vogel and Davies, 1991), even in neurofibromin-deficient neurons. We had previously reported that neurotrophins promote the survival of subpopulations of *Nf1*^{-/-} DRG and trigeminal sensory neurons, but only for those ganglia isolated at later stages of development (E12.5, E13); NGF has no effect on

DRG neurons isolated from E11.5 *Nf1* mutant embryos, for example (Vogel et al., 1995; K.S. Vogel, unpublished results). To begin to address this question, we performed NGF withdrawal experiments on DRG neurons isolated from E12.0 *Nf1*^{-/-}, *Nf1*^{+/-}, and *Nf1*^{+/+} mouse embryos. We found that neurons of all three genotypes, isolated at this slightly earlier stage, were more resistant to the apoptosis-inducing effects of NGF removal, particularly after 72 hours in the presence of this neurotrophin (**Figure 5**).

Based on the above, and other unpublished results, we propose that target contact, and concomitant exposure to neurotrophins, initiates the development of neurotrophin dependence in peripheral neurons, even if they lack neurofibromin. Environmental cues encountered by growing axons *en route* to the target undoubtedly influence acquisition of neurotrophin dependence; in cultures of ganglia isolated from developing embryos, some neurons may have extended axons towards or even contacted the peripheral target, whereas others have not yet developed axons. We reported previously that SCG neurons isolated from *Nf1*^{-/-} embryos never develop neurotrophin dependence *in vitro*, regardless of NGF exposure and withdrawal paradigms (Vogel et al., 1995). This may reflect the fact that few, if any, SCG neurons isolated at E13.5 (the latest stage to which *Nf1*^{-/-} mouse embryos survive) have extended axons towards peripheral targets, and certainly none have contacted their targets *in vivo*. Our hypothesis on the role of environmental cues and target contact in the development of neurotrophin dependence can be tested experimentally by manipulating neuron/target interactions in explant co-cultures, and the PI is preparing a grant proposal to obtain additional funding for this project. With respect to the current award, we plan to expand the NGF withdrawal experiments shown in Figures 4 and 5 to include DRG neurons isolated from E11.5 *Nf1*^{-/-}, *Nf1*^{+/-}, and *Nf1*^{+/+} mouse embryos, and to examine markers of apoptotic events (phosphorylated c-jun expression, cytochrome c release, etc.) in these cultures. The other type of experiment that can yield useful data in a short period of time is to plate *Nf1*^{-/-} DRG neurons in the absence of neurotrophins, expose them to NGF for defined periods of time *in vitro*, and then remove the neurotrophin and monitor survival. In combination with the KCl rescue experiments described in the next paragraph, these data should form the basis for a manuscript to be submitted to Journal of Neuroscience, or Journal of Neurobiology.

Because we did not expect the *Nf1* mutant DRG neurons to undergo apoptosis following NGF removal, we predicted that depolarizing levels of potassium would have no effect on the survival of neurofibromin-deficient neurons. However, we found that 40mM KCl rescued almost all E12.5 *Nf1*^{-/-} DRG neurons following NGF withdrawal at 24 hours *in vitro*; in the absence of KCl, half of the *Nf1* mutant neurons died within 24 hours of NGF removal (**Figure 6**). **Figure 6** illustrates a typical response of young (E12.5) *Nf1*^{+/+} and *Nf1*^{+/-} DRG neurons to NGF withdrawal, and even high levels of KCl (40 mM) cannot rescue all of the deprived neurons. 50 mM KCl appears to be too high a dose, and in fact may kill *Nf1*^{-/-} DRG neurons at this stage (**Figure 6**). This unexpected result with KCl rescue of neurofibromin-deficient neurons requires that we identify optimal concentrations of extracellular potassium for E12.5 *Nf1*^{-/-} DRG neurons, and compare with those required to rescue neurons isolated from *Nf1*^{+/-} and *Nf1*^{+/+} littermates. We also propose to combine these experiments with those outlined in Task 2, in order to characterize the signaling mechanisms for KCl rescue in the context of neurofibromin deficiency.

Retinal Neurons. We encountered the same difficulties with E12.5 retinal cultures as described above for E13 and E15 retinae (Task 1). As an alternative neuron type for the experiments in Task 4, I favor trigeminal ganglion sensory neurons for the following reasons. First, we know that some E12 and E13 trigeminal neurons isolated from *Nf1*^{-/-} embryos are dependent on NGF, whereas *Nf1* mutant SCG neurons isolated at the latest stage (E13.5) never exhibit any response to NGF (Vogel et al., 1995). Second, the time course of target contact and the development of neurotrophin dependence have been well-characterized for trigeminal neurons in wild-type embryos. We have demonstrated that trigeminal ganglion neurons extend axons to the whisker pad (maxillary process) in a grossly normal pattern in *Nf1*^{-/-} mouse embryos (K.S. Vogel and L.F. Parada, unpublished data). Finally, the target interactions of trigeminal ganglion neurons can be manipulated readily in explant co-cultures, and ganglia of different stages and genotypes can be exposed to heterotypic and heterochronic target tissues.

Task 5. Characterize the time course of apoptotic events at the mitochondrial level following neurotrophin withdrawal (Months 6-18)

During the first year of funding, we have optimized culture conditions on glass for the confocal microscopy of embryonic DRG neurons, by modifying milled tissue culture dishes used in Dr. James Lechleiter's laboratory to monitor astrocyte cultures. In addition, we have identified concentrations of MitoTracker dyes that allow comparison of mitochondrial morphology and localization in *Nf1*^{-/-} and wild-type DRG neurons deprived of NGF.

Tasks 6-8. (Months 12-36) N/A for first year of funding.

KEY RESEARCH ACCOMPLISHMENTS

- Optimized culture conditions and quantitative methods for the NGF withdrawal paradigm, using embryonic mouse DRG neurons (Tasks 1, 2, 4)
- Identified differences in responses to NGF withdrawal for *Nf1*^{+/+} and ^{+/-} DRG neurons isolated at E13, E15, and E17 (Task 1)
- Identified concentrations of depolarizing KCl that promote survival of DRG neurons following NGF withdrawal (Task 1)
- Characterized effects of NGF withdrawal on E12.5 *Nf1*^{-/-} DRG neurons (Task 4)

REPORTABLE OUTCOMES

Manuscripts in preparation:

Brannan, C.I., and Vogel, K.S. (in preparation) Increased neurotrophin sensitivity and delayed apoptosis in sensory and sympathetic neurons that lack an alternatively spliced form of neurofibromin. *To be submitted to the Journal of Neuroscience, or Neuron*

Vogel, K.S. (in preparation) *Nf1* haploinsufficiency alters the response to NGF withdrawal and depolarization in sensory neurons. *To be submitted to the Journal of Neuroscience.*

Employment and Training Opportunities:

Tyler Grass was hired as a laboratory assistant at the beginning of June 2003 to perform immunocytochemical and pharmacological analyses for the proposed research. He performed much of the work presented in Table 2 of the Appendices, and also examined expression of phosphorylated c-jun in DRG neurons. Tyler will start college this fall, and is interested in

neuroscience research; I would like to continue to employ him part-time on this grant. In addition, I plan to hire a research assistant at the master's degree level, to fill the vacant graduate student position on this project.

CONCLUSIONS

Importance and Implications. Our results to date support the emerging idea that neurofibromin expression and *Nf1* haploinsufficiency influence the behavior of both peripheral and central neurons. Loss of neurofibromin, with the resulting abnormalities in Ras and PI3 kinase signaling, has profound effects on the neurotrophin dependence and sensitivity of embryonic sensory neurons (Vogel et al., 1995; Klesse and Parada, 1998; Vogel et al., 2000). Behavioral experiments with *Nf1*^{+/-} and *exon23a*^{-/-} mice indicate that neurofibromin function in CNS neurons modulates learning and memory (Silva et al., 1997; Costa et al., 2001). The experiments completed for Task 1 are consistent with a role for neurofibromin in modulating both the apoptotic response to NGF withdrawal, and the survival response to depolarizing levels of potassium. Absence of neurofibromin does not guarantee protection from apoptosis, however, as the results for Task 4 indicate that some *Nf1*^{-/-} neurons die following NGF removal. These findings have allowed us to develop new hypotheses regarding the influences of target contact, target-derived cues, and neurofibromin levels on acquisition of neurotrophin dependence in developing sensory neurons.

Changes to the Research Plan.

- For Tasks 1 and 4, I propose to use either SCG sympathetic neurons or trigeminal sensory neurons, rather than retinal neurons. The difficulties encountered with embryonic retinal neurons are described under the Task 1 report.
- The experiments described in Tasks 2 and 4 were not completed during Year 1, primarily due to the unexpected finding that some *Nf1*^{-/-} DRG neurons die following NGF withdrawal. We plan to obtain additional funding to address the role of target interaction, but in the context of this study we would like to include *Nf1* mutant neurons in the pharmacological experiments for Task 2. In addition, we propose to analyze the effects of different NGF exposure and withdrawal patterns for Task 4.

"So What" Section. The learning disabilities associated with NF1 constitute a highly variable phenotype, and in addition represent a controversial topic of research and clinical interpretations. Using mice that harbor targeted mutations in *Nf1*, Silva and colleagues have demonstrated that aberrant or reduced regulation of Ras signaling by neurofibromin may contribute to certain aspects of the spatial learning disorder (Silva et al., 1997; Costa et al., 2001). More recently, these researchers have proposed that the excessive Ras activity in *Nf1*^{+/-} neurons leads to increased GABA-mediated inhibition and defects in long-term potentiation (Costa et al., 2002). Our results in Task 1 are consistent with the interpretation that *Nf1*^{+/-} neurons may respond aberrantly to electrochemical (ion gradients) and neurotrophin stimuli, which could potentially affect neuronal function and synaptic transmission. The experiments outlined in Task 2 will allow us to address the specific signaling pathways downstream of Ras and neurofibromin that are involved in these responses.

Our results may also contribute to the understanding of the mechanisms of apoptosis in sensory neurons. Most of the molecular studies of apoptosis have focused on neonatal rat SCG

sympathetic neurons, and for the experimental paradigm of NGF withdrawal/KCl rescue, there is disagreement regarding the role of the PI3 kinase/Akt pathway in mediating the survival response to depolarization. Crowder and Freeman (1998) reported that activation of PI3 kinase and Akt is necessary and sufficient for the survival of NGF-deprived SCG neurons; Vaillant and colleagues (1999) found that both depolarization and neurotrophins promote SCG neuron survival through regulation of PI3 kinase/Akt signaling. In contrast, Tsui-Pierchala and colleagues (2000) found that PI3 kinase inhibition does not block the survival-promoting effects of NGF. We have shown that, although loss of neurofibromin does not protect all DRG neurons from apoptosis following NGF withdrawal, it does increase the sensitivity to rescue by depolarization. Results with embryonic mouse (Klesse and Parada, 1998) and rat (Vogelbaum et al., 1998) DRG neurons indicate that PI3 kinase/Akt signaling is essential for NGF-mediated survival, and the experiments outlined in Task 2 will allow us to define the role of this pathway in **depolarization**-mediated survival. Both neurotrophin signaling and activity-mediated processes are required to achieve correct target innervation patterns and synaptic plasticity in the peripheral nervous system.

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APPENDICES

Figure Legends

Figures 1A, 1B: Responses of E13 *Nf1*^{+/+} and *Nf1*^{+/-} DRG neurons to NGF withdrawal and KCl rescue. Dissociated DRG neurons were plated at low density (200-800 neurons/35mm dish) in the presence of 2.5S NGF (5 ng/ml) for 24 or 72 hours. NGF was withdrawn from the cultures by 3 successive 40-minute washes, and replaced with fresh NGF (positive control), KCl (40 or 50 mM), or no neurotrophins (negative control). Each bar represents the average of counts from 3 or more different cultures; error bars represent *ranges* of values obtained.

Figures 2A, 2B: Responses of E15 *Nf1*^{+/+} and *Nf1*^{+/-} DRG neurons to NGF withdrawal and KCl rescue. Neuronal cultures were prepared and NGF withdrawn as described for Figure 1. Each bar represents the average of counts from 3 or more different cultures; error bars represent *ranges* of values obtained.

Figures 3A, 3B: Responses of E17 *Nf1*^{+/+} and *Nf1*^{+/-} DRG neurons to NGF withdrawal and KCl rescue. Neuronal cultures were prepared and NGF withdrawn as described for Figure 1. Each bar represents the average of counts from 3 or more different cultures; error bars represent *ranges* of values obtained.

Figure 4: Responses of E12.5 *Nf1*^{+/-} and *Nf1*^{-/-} DRG neurons to NGF withdrawal. Neuronal cultures were prepared and NGF withdrawn as described for Figure 1. Each bar represents the average of counts from 2 or more different cultures; error bars represent *ranges* of values obtained.

Figure 5: Responses of E12.0 *Nf1*^{+/+}, *Nf1*^{+/-}, and *Nf1*^{-/-} DRG neurons to NGF withdrawal. Neuronal cultures were prepared and NGF withdrawn as described for Figure 1. Each bar represents the average of counts from 2 or more different cultures.

Figure 6: Responses of E12.5 *Nf1*^{+/+}, *Nf1*^{+/-}, and *Nf1*^{-/-} DRG neurons to NGF withdrawal and KCl rescue. Neuronal cultures were prepared and NGF withdrawn as described for Figure 1. Each bar represents the average of counts from 2 or more different cultures.

Table 1. Increases in retinal neuron number with time in culture. Neural retinae were isolated from E15 mouse embryos, dissociated, and plated on a laminin substratum in the presence of brain-derived neurotrophic factor (BDNF, 2 ng/ml). Counts were performed at 24-hour intervals.

Table 2. Expression of neuronal markers in E15 retinal cultures in the presence and absence of BDNF. Retinal cultures on laminin were fixed after 96 hours *in vitro*, and processed for immunocytochemistry. Cultures 1 and 2 had no BDNF, whereas cultures 3 and 4 contained BDNF at 2 ng/ml.

Figure 1A.
KCl Rescue Following NGF Deprivation
at 24 Hours In Vitro: Nf1+/+ vs. Nf1+/-
E13.5 DRG Neurons

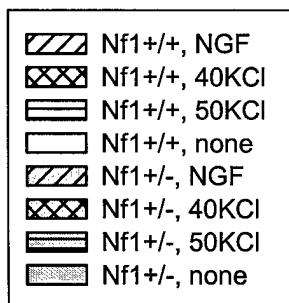
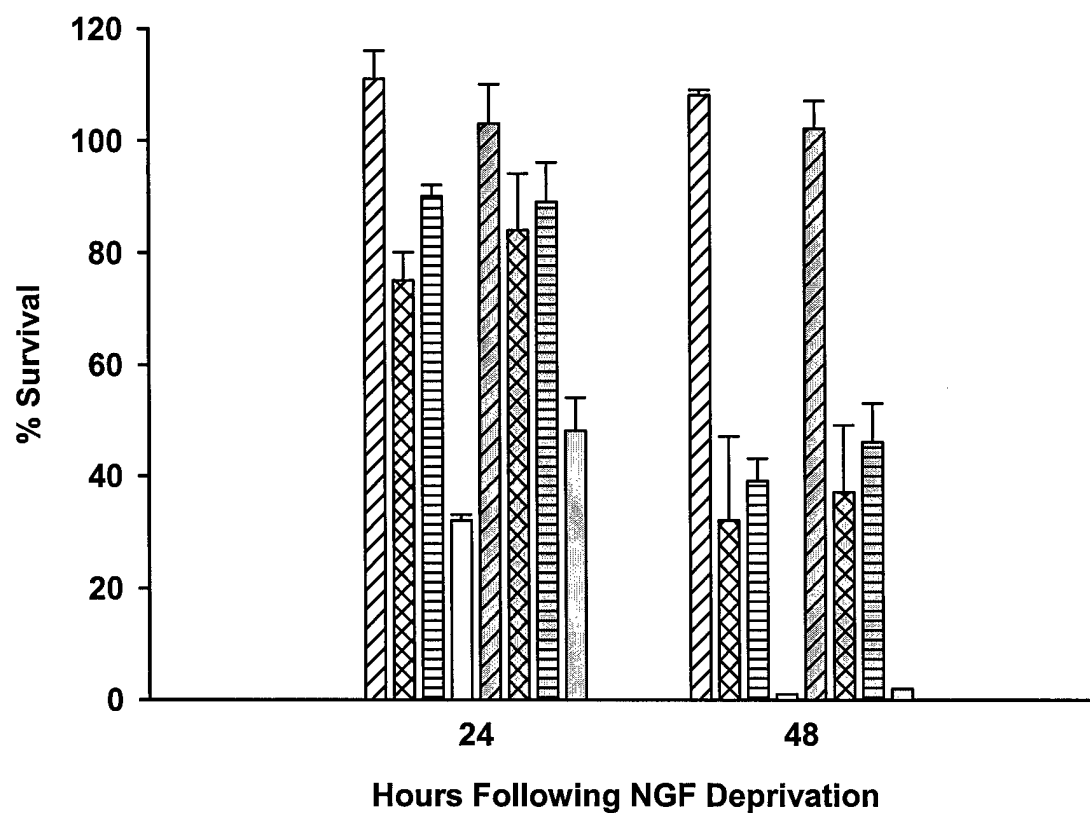


Figure 1B.
KCl Rescue Following NGF Deprivation
at 72 Hours In Vitro: Nf1+/+ vs. Nf1+/-
E13.5 DRG Neurons

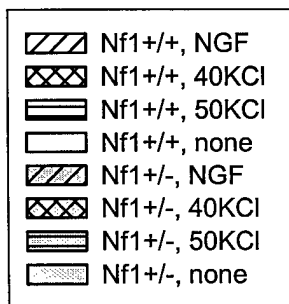
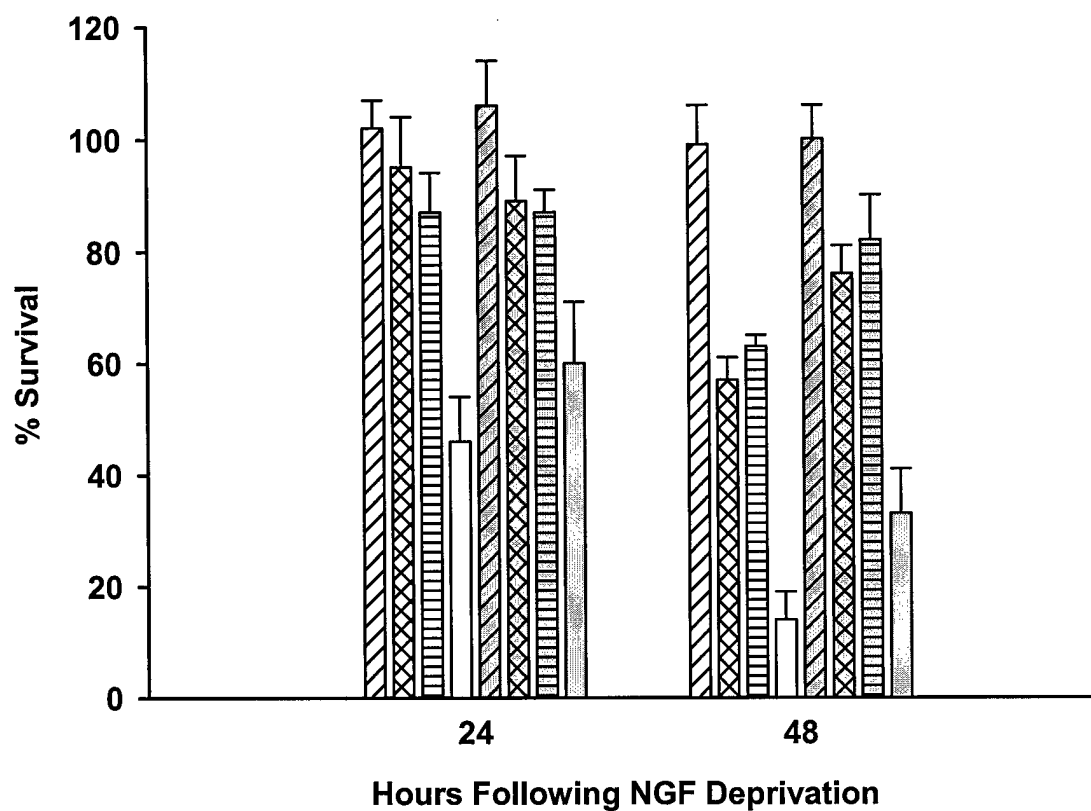


Figure 2A.
KCI Rescue Following NGF Deprivation
at 48 Hours In Vitro: Nf1+/+ vs. Nf1+/-
E15.5 DRG Neurons

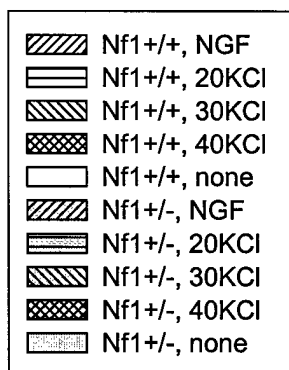
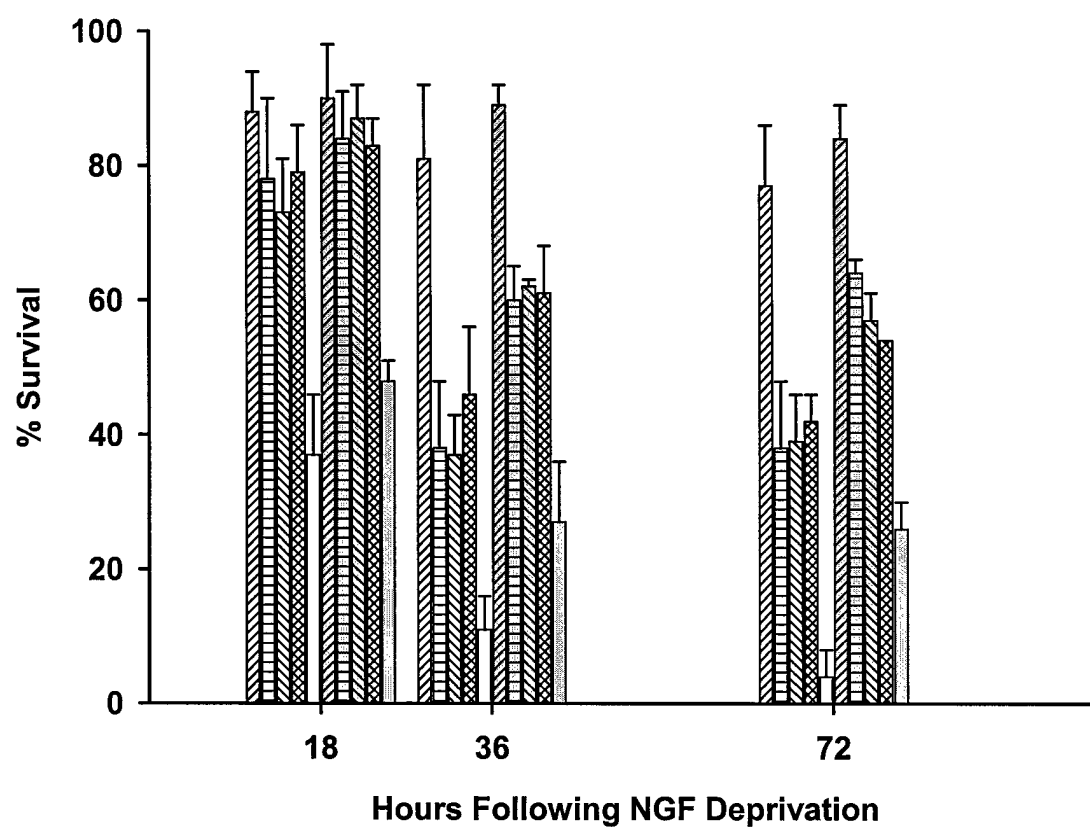


Figure 2B.
KCl Rescue Following NGF Deprivation
at 72 Hours In Vitro: Nf1+/+ vs. Nf1+/-
E15.5 DRG Neurons

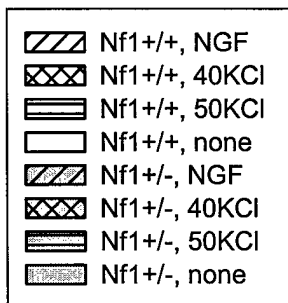
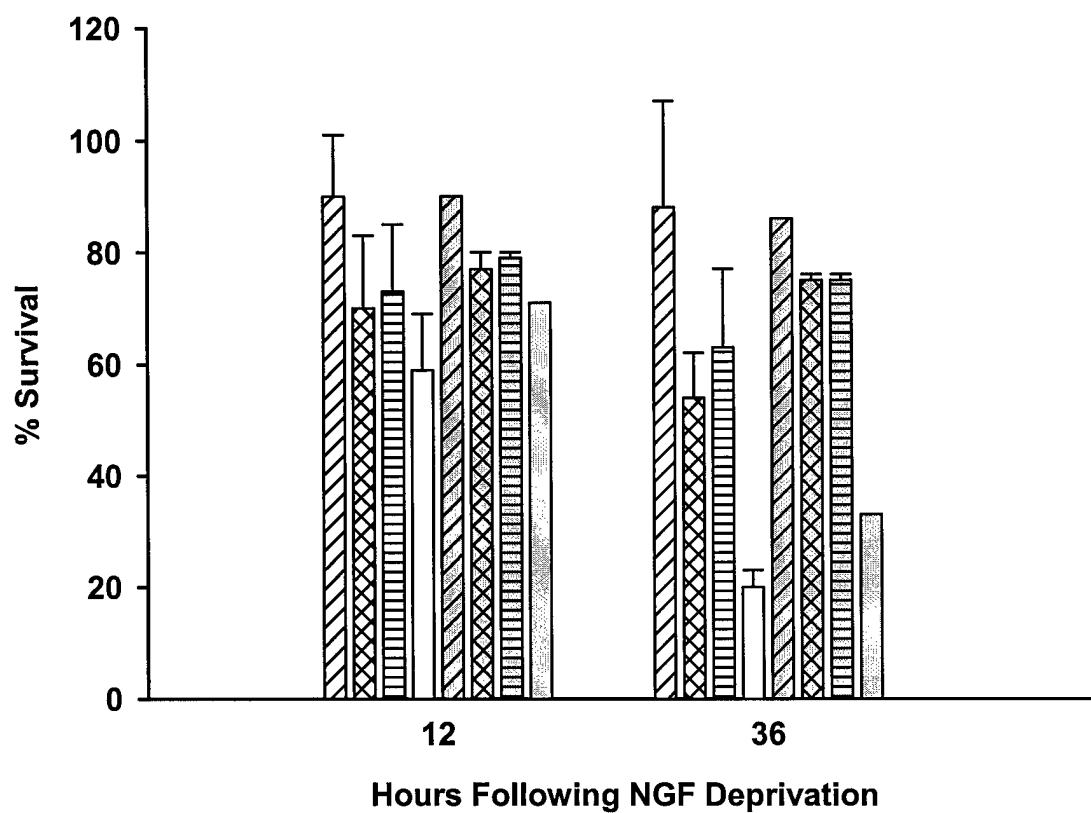


Figure 3A.
KCl Rescue Following NGF Deprivation
at 24 Hours *In Vitro*: Nf1+/+ vs. Nf1+/-
E17 DRG Neurons

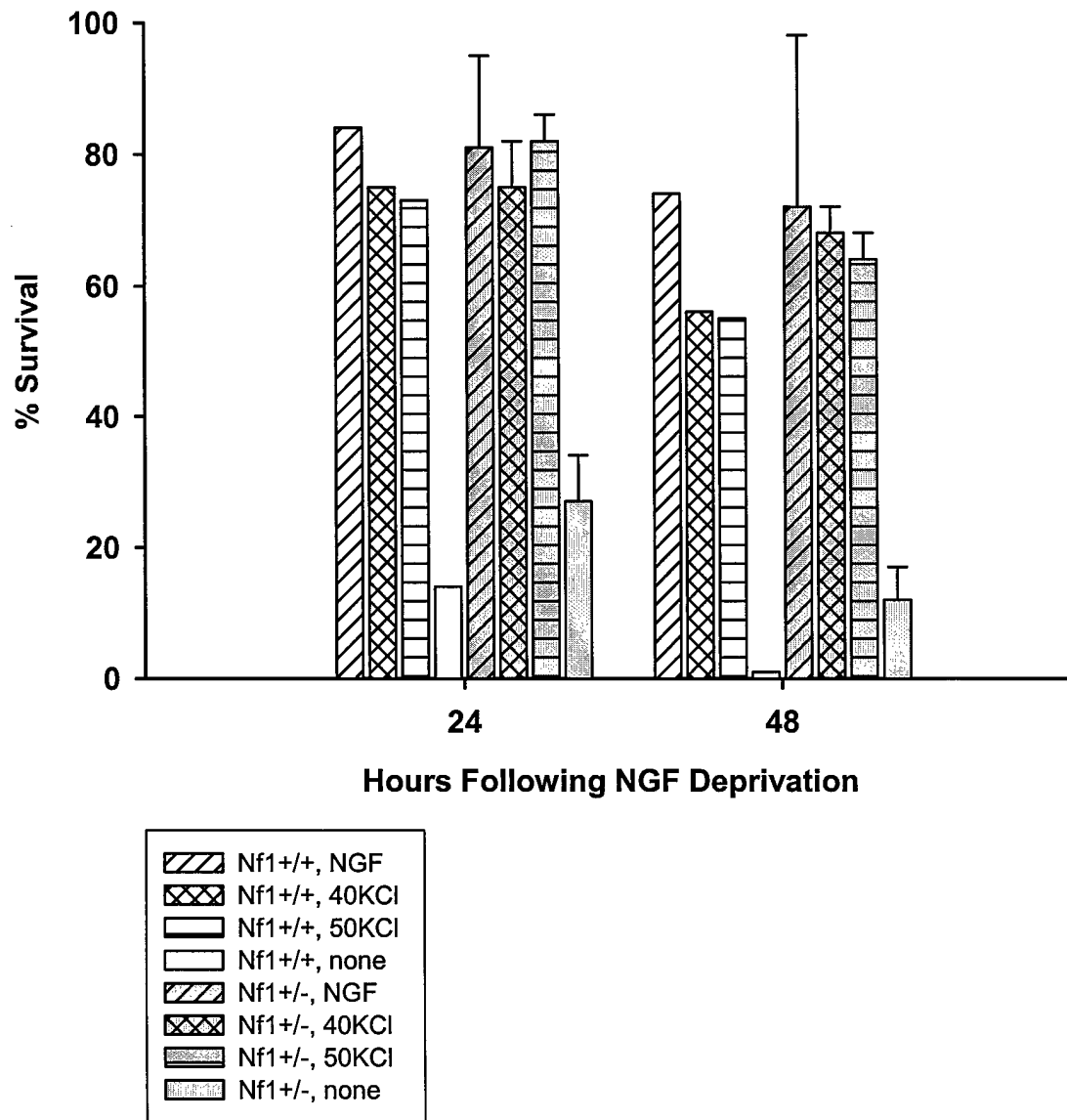


Figure 3B.
KCl Rescue Following NGF Deprivation
at 72 Hours *In Vitro*: Nf1+/+ vs. Nf1+/-
E17 DRG Neurons

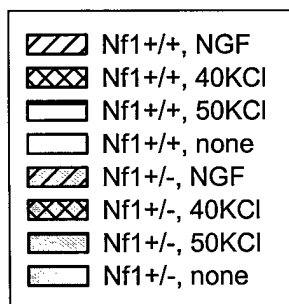
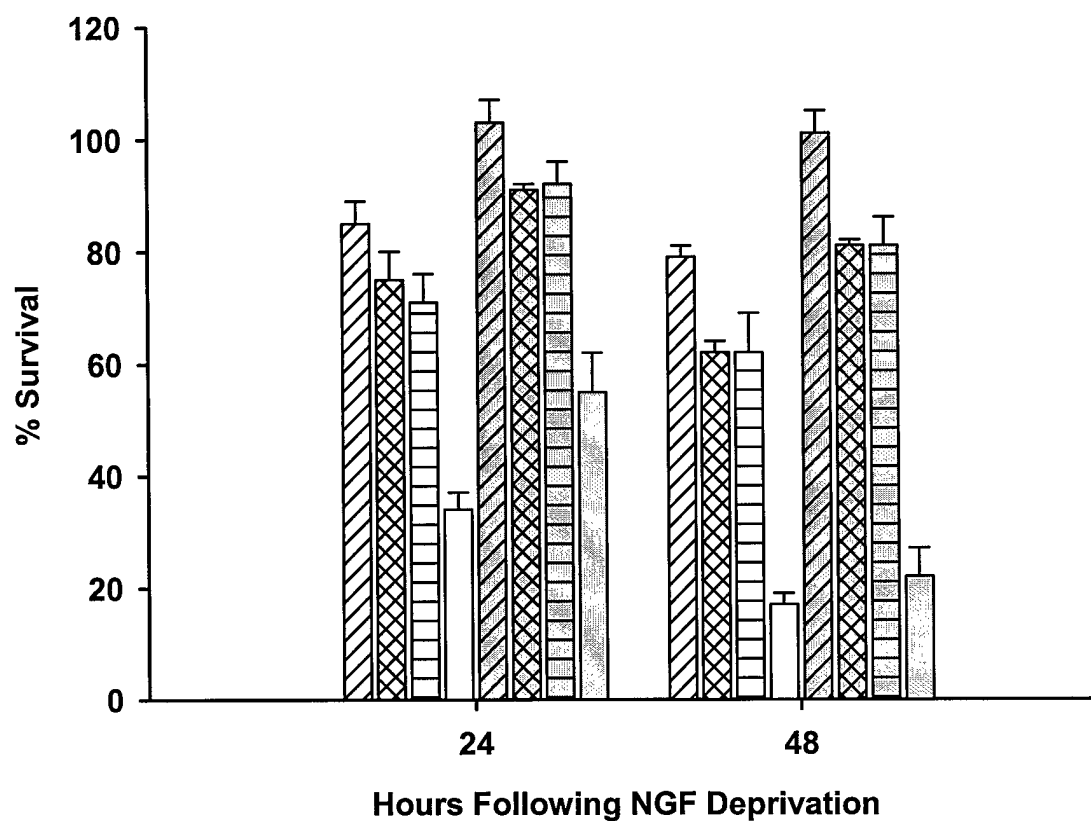


Figure 4.
Survival Following NGF Withdrawal at 24 and 72
Hours *In Vitro*: Nf1+/- vs. Nf1-/- E12.5 DRG Neurons

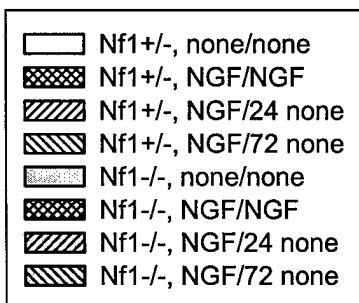
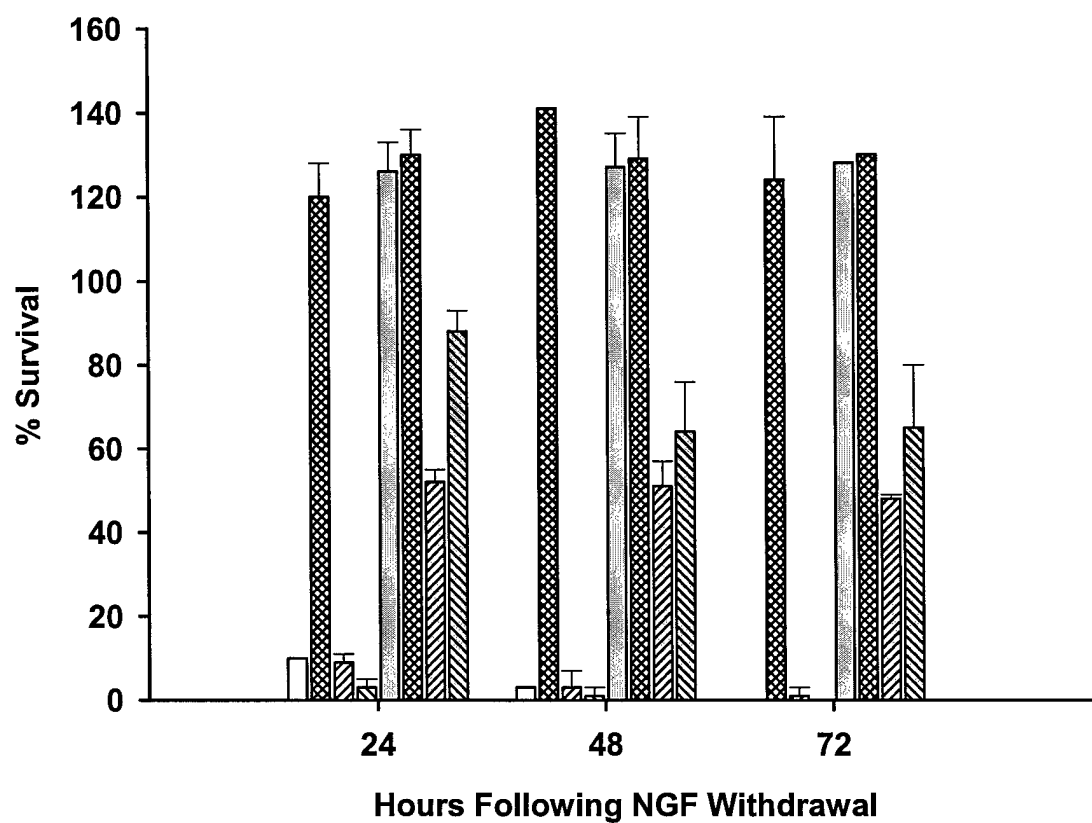


Figure 5.
Survival Following NGF Withdrawal at 24 and 72
Hours In Vitro: Nf1+/+ vs. Nf1+/- vs. Nf1-/-
E12.0 DRG Neurons

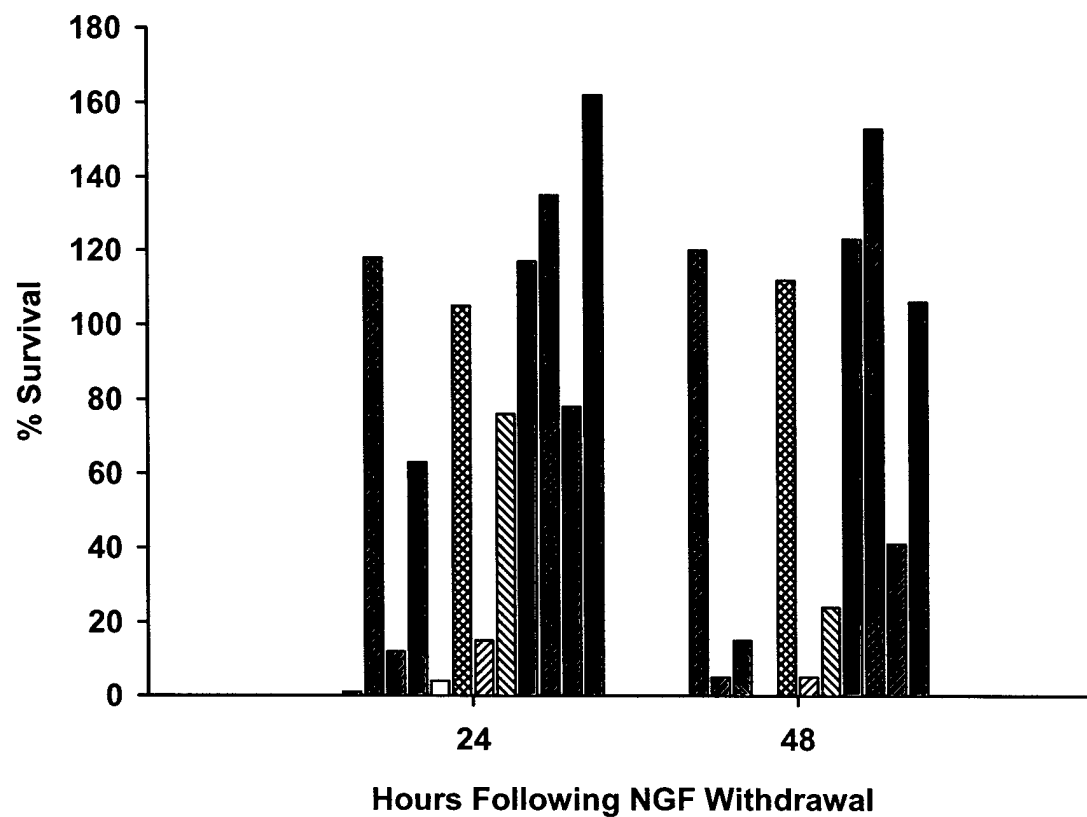


Figure 6.
KCl Rescue Following NGF Deprivation at 24 Hours
In Vitro: Nf1+/+ vs. Nf1+/- vs. Nf1-/-
E12.5 DRG Neurons

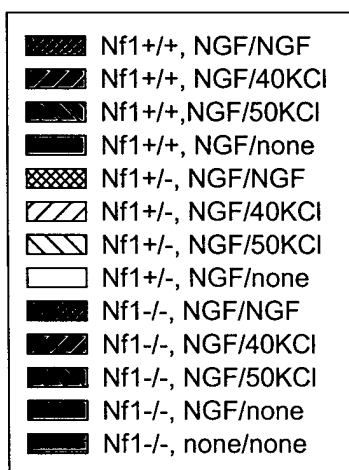
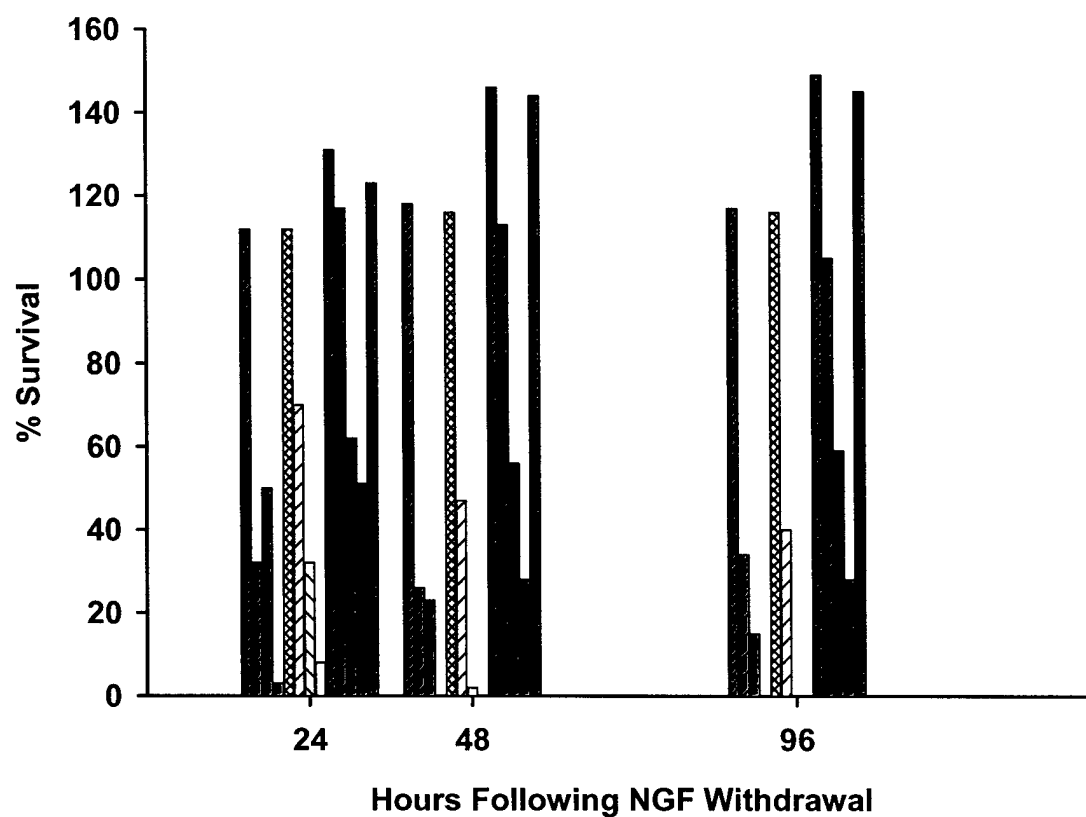


Table 1. Increase in retinal neuron number with time in vitro (E15.5, Nf1+/+)

Embryo ID	Culture #	Number of Neurons			
		24 hours	48 hours	72 hours	96 hours
NEU67A	1	36	252		343
	2	40	260		532
	3	40	337		719
	4	47	389		714
	5	49	344		635
NEU67D	1	185		832	
	2	91		532	
	3	220		800	
	4	188		971	
	5	153		1240	

Table 2. Immunostaining of E15.5 retinal cultures (Nf1+/+)

Culture #	% Immunopositive Cells			
	Synapsin	Neurofilament-Med	Calretinin	GAP-43
1	71	35	74	85
2	60	43	87	87
3	61	55	82	87
4	73	60	80	71